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Cell cycle genes regulate *vestigial* and *scalloped* to ensure normal proliferation in the wing disc of *Drosophila melanogaster*

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Abstract
In *Drosophila*, the Vestigial-Scalloped (VG-SD) dimeric transcription factor is required for wing cell identity and proliferation. Previous results have shown that VG-SD controls expression of the cell cycle positive regulator *dE2F1* during wing development. Since wing disc growth is a homeostatic process, we investigated the possibility that genes involved in cell cycle progression regulate *vg* and *sd* expression in feedback loops. We focused our experiments on two major regulators of cell cycle progression: *dE2F1* and the antagonist *dacapo* (*dap*). Our results reinforce the idea that VG/SD stoichiometry is critical for correct development and that an excess in SD over VG disrupts wing growth. We reveal that transcriptional activity of VG-SD and the VG/SD ratio are both modulated upon down-expression of cell cycle genes. We also detected a *dap*-induced *sd* upregulation that disrupts wing growth. Moreover, we observed a rescue of a *vg* hypomorphic mutant phenotype by *dE2F1* that is concomitant with *vg* and *sd* induction. This regulation of the VG-SD activity by *dE2F1* is dependent on the *vg* genetic background. Our results support the hypothesis that cell cycle genes fine-tune wing growth and cell proliferation, in part, through control of the VG/SD stoichiometry and activity. This points to a homeostatic feedback regulation between proliferation regulators and the VG-SD wing selector.

Introduction

Cell proliferation is a complex process that requires the activity of the genes involved in cell cycle progression, DNA replication and mitosis. Previous work has provided evidence that differentiation or patterning genes directly control cell cycle gene expression (Duman-Scheel *et al.* 2002; Hwang *et al.* 2002). Conversely, proliferation signals also regulate various differentiation pathways (Muller *et al.* 2001; Dimova *et al.* 2003).

The *Drosophila* wing imaginal disc provides a powerful model for investigating the pathways that connect growth, proliferation and patterning during development (Neufeld *et al.* 1998). Genes of the E2F family are the main regulators of proliferation and cell cycle control (Harbour & Dean 2000). The binding of dE2F1 to the DP subunit provides a heterodimeric transcription factor whose target genes ensure the G1/S transition (Ohtani & Nevins 1994; Duronio *et al.* 1995). During the G1 phase, expression of these genes is repressed by the binding of the

RBF-related pocket proteins (Retinoblastoma Family) to dE2F1. At late G1, RBF phosphorylation by Cyclin-CDK complexes releases free dE2F1-DP which up-regulates genes involved in the DNA replication machinery as well as cell cycle progression (Du *et al.* 1996). In *Drosophila,* the Cyclin-dependent Kinase Inhibitor (CKI) *dacapo* (*dap*, a $p21^{CIP1}$ homolog) inhibits the core cell cycle machinery through the binding and down-regulation of Cyclin-CDK enzymatic activities. This restriction of the G1/S transition often accompanies the switch from proliferation to differentiation (de Nooij *et al.* 1996; Lane *et al.* 1996). Recently, reports identified respective feedback regulations involving *dE2F1* and *dap* expression that prevent unbalanced cell cycle progression (Reis & Edgar 2004).

The *Drosophila* wing originates during embryogenesis from a primordium of cells that proliferate rapidly and homogeneously until the end of the third instar. Growth of the wing disc is patterned by the establishment of Anterior/Posterior (A/P) and Dorsal/Ventral (D/V) compartments that define two axes of the wing (Blair 1995).

The *vestigial* (*vg*) gene is one of the main targets defined by these two axes. *vg* encodes a nuclear protein expressed in the wing presumptive region (wing pouch) of the imaginal disc (Williams *et al.* 1991). All *vg* homozygous mutants are characterized by a wing phenotype and heterozygotes display nicks in the wings with a weak penetrance (Goux & Paillard 1976). A complete absence of wing structures is observed in vg^{null} homozygotes (Paumard-Rigal *et al.* 1998; Zider *et al.* 1998). During larval development, *vg* expression in the wing disc relies on the sequential activation of two intronic enhancers. The boundary enhancer (vg^{BE}) is activated at mid-second larval instar and mediates the transcription of *vg,* at the D/V boundary, under the direct control of the Notch (N) pathway. At the beginning of third instar, the quadrant enhancer (vg^{QE}) directs *vg* expression in the remainder of the wing pouch. Thus, *vg* integrates inputs from the two axes that control patterned wing development (Williams *et al.* 1994; Kim *et al.* 1996).

In the wing pouch, the VG and Scalloped (SD) proteins colocalize and interact molecularly. The VG-SD dimer is a functional transcription factor, in which VG provides the transcription activator function, while SD binds DNA *via* its Transcriptional Enhancer Activator (TEA) domain (Halder *et al.* 1998; Simmonds *et al.* 1998; Vaudin *et al.* 1999).

Previous results have suggested that VG-SD is required for wing growth and cell proliferation (Martin-Castellanos & Edgar 2002; Kolzer *et al.* 2003). vg^{null} cell clones do not proliferate in the wing pouch and ectopic expression of *vg* in other imaginal discs induces ectopic proliferation of wing tissue (Kim *et al.* 1996). Moreover, *vg* induces cell cycle progression from G1 to S and G2 to M phases, through the activation of key genes involved in proliferation, such as *dE2F1* and the G2/M transition regulator *string* (yeast *cdc25* homolog) (Delanoue *et al.* 2004). Since VG-SD controls cell proliferation and growth of the wing, we tested the hypothesis that feedback loops link cell cycle genes to the VG-SD dimer. Such homeostatic regulations would, in turn, ensure correct growth and proliferation. To address this issue, we assessed the possible effect of two proliferation regulators, *dE2F1* and the antagonist *dap,* on the regulation of VG-SD activity. In this study, the critical requirement for exquisite VG/SD stoichiometry regulation is confirmed. In addition, our results demonstrate that respective expressions of *vg* and *sd*, as well as VG-SD activity, are finely tuned according to feedback loops that connect cell cycle genes and the VG-SD dimer *in vivo*.

Results

The ratio of SD over VG is critical for wing growth

vg and *sd* mutants display strong wing mutant phenotypes (fig 1A) and VG-SD dimerization is required for normal wing formation. Moreover, ectopic *vg* induces *sd* expression (Halder *et al.* 1998; Simmonds *et al.* 1998). In addition, SD DNA target selectivity is modified *in vitro* when VG is dimerized with SD (Halder & Carroll 2001), suggesting that an unbalanced VG/SD ratio

would significantly modify the expression of VG-SD targets. Previous results have shown that *sd* over-expression is deleterious for endogenous wing growth (Simmonds *et al.* 1998). Therefore, to understand the physiological relevance of the VG/SD ratio during development, it was important to evaluate the effect of VG/SD imbalance on wing growth and development, by using both mutants and over-expression of *vg* or *sd*.

In order to increase SD over VG we over-expressed *sd* in the *vg* expression domain (*vg-GAL4* driver) in three different genetic contexts: sd^{58}/+, +/+ or vg^{null}/+ (fig 1B). We observed that *sd* over-expression led to a strong wing phenotype (fig 1B and (Simmonds *et al*. 1998). This wing phenotype was further enhanced upon VG reduction yielding up to 93% of extreme phenotypes (fig 1B) but, was reduced in an *sd* hypomorphic context with only 5% of extreme phenotypes. The data are in accordance with the hypothesis that an increased ratio of SD over VG is deleterious for normal expression of the dimer target genes involved in wing development. Conversely, *vg* over-expression did not induce any obvious developmental defects and wings remained completely normal (data not shown), demonstrating that an increased VG/SD ratio does not have a dominant negative effect on VG-SD function in the wing pouch. Thus, correct development and growth of the *Drosophila* wing disc implies a precise regulation of the respective expressions of SD and VG *in vivo*. These results suggest, as previously hypothesized by (Simmonds *et al.* 1998), that in physiological conditions, genes involved in wing growth (*e.g.* cell cycle genes) may fine-tune SD and VG expression levels to regulate wing growth and cell proliferation.

Cell cycles genes regulate VG-SD transcriptional activity

In the wing disc, wing pouch growth is known to be a highly homeostatic process (de la Cova *et al.* 2004; Reis & Edgar 2004). The ability of VG-SD to drive proliferation and cell cycle progression largely relies on the regulation of dE2F1 and its target genes by the dimer (Delanoue *et al.* 2004). So far, however, the effects of cell cycle regulators on VG-SD activity in feed back loops have not been investigated. To assess the potential action of cell cycle genes on the VG-SD dimer, we tested the effect of reducing the dosage of two antagonist regulators, *dE2F1* and *dap,* on the dimer, using a VG-SD transcriptional activity reporter strain (fig 2). This strain contains a sensor transgene, '*hsp70-GAL4$_{db}$-sd',* in which the *sd* TEA DNA binding domain has been replaced by the GAL4 DNA binding domain. After a heat shock, a *UAS-LacZ* reporter gene is expressed only where SD::GAL4 dimerizes with a transcriptional co-activator (*e.g.* VG) (Vaudin *et al.* 1999). This strain thus provides a sensitive tool for observing VG-SD activity in the wing pouch. As a first positive test, we evaluated the effect of a VG decrease on VG-SD activity. Indeed, in vg^{null}/+ wing discs, a significant down-regulation of the VG-SD activity reporter construct was observed compared to control (fig 2A, B); *vg* is therefore not fully haplo-sufficient. In addition, we performed quantitative real time RT-PCR experiments and measured *vg* and *sd* transcript expression in vg^{null} heterozygous wing discs (fig 2E). Compared to the w^{1118} control strain (*vg* and *sd* arbitrary levels = 1), we observed a significant decrease in the amount of *vg* (0.38 ± 0.05), whereas the level of *sd* remained comparable to that in the wild type strain (0.96 ± 0.07), leading to a decrease in the *vg/sd* ratio. The diminution of the dimer activity observed (fig 2B) is therefore likely due to competition between the SD::GAL4 chimeric construct and endogenous SD for a lower amount of the available VG transcriptional activator. Conversely, in a sd^{1}/+ heterozygote background, VG-SD activity was greater than that in the control, probably due to a lower quantity of endogenous SD protein for the same amount of VG and SD::GAL4 protein (data not shown). This is in agreement with the hypothesis that a precise VG/SD stoichiometry is required during wing development.

To monitor the effects of cell cycle regulators on VG-SD, experiments with the reporter construct were carried out in heterozygous

dE2F1 or *dap* null mutants, which display no wing phenotypes (Duronio *et al.* 1995). VG-SD activity was significantly reduced in heterozygotes for the $dE2F1^{91}$ amorph allele (fig 2C). RT-PCR experiments revealed a decrease in *vg* (0.68 ± 0.12) and an increase in *sd* (1.20 ± 0.21) transcripts compared to control (fig 2E). This led to a significantly lower *vg/sd* ratio, most likely responsible for the reduced VG-SD reporter activity observed (fig 2C). This result demonstrates that *dE2F1* function is crucial for *vg, sd* normal expression and VG-SD activity.

Since DAP is an inhibitor of cell cycle progression, the level of VG-SD sensor activity was also assessed in heterozygotes for the dap^{04454} amorph allele (de Nooij *et al.* 1996). Although no significant modulation of VG-SD activity could be detected (fig 2A,D), RT-PCR experiments revealed a strong decrease in both *vg* (0.44 ± 0.01) and *sd* (0.36 ± 0.07) expression levels compared to control. Thus, *dap* is required for normal *vg* and *sd* expression. Despite the reduced *vg* and *sd* levels, the *vg/sd* ratio remained similar to the control in this case (fig 2E), and VG-SD activity was thus unmodified. Therefore, to some extent, the *vg/sd* ratio, rather than absolute expression levels of *vg* and *sd*, directs reporter strain and VG-SD activities. Moreover, genetic interactions showed that both VG-SD activity and a normal *vg/sd* ratio were greatly restored, in the $dE2F1^{91}$/+; dap^{04454}/+ double heterozygotes (data not shown). Our results demonstrate that cell cycle gene down-expression modulates expression of *vg* and *sd* and VG-SD activity. Moreover, *dap* and *dE2F1* loss of function display opposite effects on VG-SD activity and, strikingly, on *sd* expression. As a whole, this sheds light on a process of feedback regulation between cell cycle regulators and VG-SD.

Ectopic *dap* induces *sd* and disrupts VG-SD activity

We next investigated whether the modulation of the respective expressions of *vg* and *sd* by cell cycle genes affects the regulation of wing disc growth and cell proliferation. We observed the strong requirement of *dap* for both *vg* and *sd* normal expression (see above and fig 2). The next step was to test how *dap* intervenes in the process. The role of DAP protein in G1 arrest has been clearly demonstrated in *Drosophila* and *dap* ectopic expression induces cell cycle arrest without triggering apoptosis (Lane *et al.* 1996; Van de Bor *et al.* 1999). *dap* over-expression along the wing D/V boundary decreases proliferation and induces nicks at the wing margin (Delanoue *et al.* 2004). Accordingly, using the *patched* (*ptc*)*-GAL4* driver, we found that ectopic *dap* restricts G1/S transition in BrdU pulse experiments (fig 3D), and decreases the number of *ptc*-expressing cells in the wing disc (fig 3A,C), and the adult wing blade (fig 3B,C).

To evaluate whether this cell cycle arrest was associated with VG-SD impairment, we tested the possible effect of over-expressing *dap* on the expression of VG and SD. Since no anti-SD antibodies are currently available, we used the sd^{ETX4} strain, a *P[lacZ]* enhancer trap (see fig 6A). Ectopic *dap*, along the *ptc* domain, had no effect on VG (data not shown), but ectopically activated *sd* expression (fig 3E). This *sd* up-regulation was strong in the wing pouch, but was also triggered in hinge and notum regions (fig 3F).

Since *sd* induction in response to *dap* correlates with a G1-S transition delay and a decrease in cell proliferation (fig 3A-D), we tested the functional significance of this VG/SD imbalance.

We hypothesized that, if *dap* slows down proliferation by inducing *sd,* thus decreasing the VG/SD ratio, this might result in a wing phenotype that should be strengthened by further VG reduction. We tested this hypothesis by over-expressing *dap* at the margin (*vg-GAL4* driver) of wild type and vg^{null}/+ wings. As expected, nicks were observed in a wild type context (fig 4A) and this phenotype was greatly enhanced in *vg* heterozygotes (fig 4B) suggesting that *dap* inhibits cell proliferation through impairment of VG/SD. Next, we tested the fate of *cut* (*ct*) at the D/V boundary of the disc as an assay of a VG-SD downstream target gene (fig 3G) (Guss *et al.*). CT expression was specifically lost in cells in which *sd* was induced in response to *dap* (fig 3H). Both *ct* and

wingless (*wg*) are also D/V-specific N targets. Nevertheless, *wg* remained unaffected, suggesting that *dap* does not affect the N pathway. We concluded that *dap* over-expression leads to *sd* induction, VG-SD dimer imbalance and impairment of its function in wing development.

Ectopic *dE2F1* rescues the vg^{83b27} mutant

The results we obtained with *dap* led us to test whether dE2F1, which is required for VG-SD normal activity and antagonizes DAP, could possibly restore wing growth in a vg^{83b27}mutant that displays no wing structures (fig1A). This mutant carries a complete deletion of the *vg* 2nd intron that spans the *vgBE* enhancer required for *vg* expression in the wing pouch, the remainder of the *vg* sequence being normal (Williams *et al.* 1994).

Ectopic expression of both *dE2F1-DP* and *P35*, a caspase inhibitor that prevents E2F-induced apoptosis (Neufeld *et al.* 1998), was monitored in a vg^{83b27} mutant, using the *vg-GAL4* driver that allowed the recovery of adults flies. dE2F1-DP expression restored growth of vg^{83b27} wing appendages, which often displayed distinct veins and margin (compare fig 5B insets and 1A). Neither the expression of P35 itself nor that of dIAP1, another caspase inhibitor, was sufficient to rescue the vg^{83b27} phenotype (Van de Bor *et al.* 1999). Consistently, dE2F1-DP induced a massive over-growth of the vg^{83b27} wing pouch (fig 5C, D) that was associated with significant proliferation. Nevertheless, in vg^{null} mutants for which the entire *vg* sequence is deleted, no such phenotypic rescue could be detected, and dE2F1-DP led to only a slight increase in hinge growth (compare fig 1A and 5A, B). This vg^{null} wing phenotype was rescued by *vg-GAL4* driven expression of *vg* as expected (data not shown). Therefore, we concluded that the partial wing rescue of the vg^{83b27} mutant (but not of the vg^{null} mutant) by dE2F1-DP, required the *vg* sequence but not the *vgBE* enhancer.

Moreover, in response to *vg-GAL4* driven dE2F1-DP expression, VG was induced in the vg^{83b27} wing pouch (fig 5F), which does not normally display either VG or SD endogenous expression (fig 5C,E and (Williams *et al.* 1991). VG induction was also obtained using the *ptc-GAL4* driver, confirming that dE2F1 is able to induce *vg* expression, even outside the wing pouch (data not shown). These results demonstrate that *vg* expression is a prerequisite for rescue of the wing phenotype in response to dE2F1.

In addition, using the sd^{ETX4} enhancer trap, we found that ectopic *dE2F1-DP* also induced *sd* expression in the wing pouch (fig 5C, G). Given the role of VG-SD in cell proliferation (Delanoue *et al.* 2004), this up-regulation of expression of both *vg* and *sd* is consistent with the wing growth rescue induced by *dE2F1*. Therefore, we tested again the expression of CT, the dimer target, upon dE2F induction, as a read-out of the dimer activity. In the vg^{83b27} mutant, no CT expression was observed at the D/V boundary (fig 5E). When *dE2F1-DP* was over-expressed, CT was induced in the wing pouch, although in a different pattern than in a wild type disc (fig 3G). In addition, we often observed ectopic proliferating wing pouches that partly matched the wing marker CT, although these morphogenetic events might not give rise to viable rescued adults, (fig 5H).

We concluded that *vg-GAL4* driven *dE2F1-DP-P35* ectopic expression, in a vg^{83b27} mutant, rescues *vg*/*sd* expression and VG-SD activity, which allow pouch cell proliferation and wing growth. All together, our results show that not only *dE2F1* is required for normal VG-SD activity in a vg^{+} background (fig 2), but that ectopic *dE2F1* rescues VG-SD activity in a *vg* hypomorphic context.

Ectopic *dE2F1* modulates *sd* expression in a homeostatic feedback loop

It has been shown that VG-SD induces *dE2F1* (Delanoue *et al.* 2004) and here, we observed that *dE2F1* can also induce VG-SD activity in a *vg* hypomorphic genetic context. Such a positive feedback loop might trigger uncontrolled wing pouch growth in a wild type context. So, since wing growth is known to proceed homeostatically, a compensatory mechanism must exist. To address this possibility, we assayed the effects of over-expressing *dE2F1-DP+P35* driven by *ptc-*

GAL4, in vg^{+} wing discs. *dE2F1* induced folding of the epithelium layer and *sd* ectopic expression along the A/P boundary but mainly in the wing pouch (fig 6A',B',C'), without any obvious effect on VG expression in vg^{+} discs (fig 6A,B), unlike previous observations in the vg^{83b27} mutant. The P35 protein *per se* had no effect. Moreover, *dE2F1* over-expression with the *vg-GAL4* driver, which allowed the recovery of adult flies, yielded nicks in the wings (fig 4C). These results demonstrate that *dE2F1* over-expression induces *sd* and decreases the VG/SD ratio, which most probably impairs the dimer function in wing development. Thus, an excess of ectopic dE2F1 in vg^{+} discs is deleterious for wing development (fig 4C), whereas, in a vg^{83b27} mutant it rescues wing growth (fig 5B).

Next, to further understand how this cross-talk depends on the *vg* genetic context, we compared the response to *dE2F1* expression in vg^{+} or vg^{null}/+. In this latter genotype, which provided an intermediate *vg* expression level (see fig 2), *sd* is less induced by dE2F1 than in vg^{+} discs (data not shown). This shows that a decrease in VG modifies the effect of ectopic *dE2F1* on *sd.* Consistently, wing nicks induced by *dE2F1* over-expression according to the *vg-GAL4 driver* (fig 4C) were partially rescued in a vg^{null}/+ heterozygote (fig 4D). Therefore, a decrease in VG attenuates the deleterious effect of over-expressed dE2F1.

Interestingly, even if in a vg^{+} background expression of both *dap* and *dE2F1* alters wing development (fig 4A,C), clear opposite behaviours are observed in vg^{null}/+ flies where the *dap* induced phenotype is enhanced, while *dE2F1* one is partially rescued (fig 4B,D). We concluded that *sd* induction and VG-SD impairment in response to *dE2F1* expression are clearly dependent on the VG expression level.

These results favour the hypothesis that sensor regulations coordinate VG-SD and cell proliferation effectors like dE2F1, and tend to ensure normal wing development. Our data suggest that *in vivo,* moderate excess in the proliferation regulator dE2F1 decreases VG-SD activity in a negative feedback loop. This may reflect a homeostatic regulation of wing growth.

Discussion

Cell proliferation relies on the tight control of cell cycle genes, and, in the wing pouch, VG-SD is also critically required. Accordingly, *vg* was shown to up-regulate *dE2F1* expression and to antagonize the CKI *dap* (Delanoue *et al.* 2004). In this study, we investigated the effects of these two antagonistic proliferation regulators in the wing pouch of the disc, and tested the hypothesis that cell cycle genes fine-tune proliferation, through regulation of the respective expressions of *vg* and *sd* and VG-SD dimer activity, thereby providing a feedback control.

VG/SD ratio and wing growth

Modulation of the relative expression levels of *vg* and *sd* as an endogenous means of fine-tuning wing growth, had previously been suggested (Simmonds *et al.* 1998). Combined loss and gain of function experiments ascertained the requirement of a precise VG/SD ratio for normal wing development and showed that an excess in SD disrupts VG-SD function in wing growth (fig 1), and probably acts as a dominant-negative through titration of functional VG-SD dimers. Therefore, *sd* induction may efficiently restrain VG-SD function *in vivo,* and a similar effect may also be physiologically achieved down-regulating *vg*. Moreover, since SD DNA target selectivity is modified upon binding of VG to SD *in vitro* (Halder & Carroll 2001), we cannot discard the hypothesis that, *in vivo* too, VG-SD targets might be different from the targets of SD alone. This could explain to some extent the phenotypes observed when *sd* is induced.

Dap induces sd and impairs VG-SD activity

Our results show that the CKI member DAP, homogeneously expressed in the wing disc (Reis & Edgar 2004), regulates VG-SD function. *dap* heterozygotes display a wild type wing phenotype, reduced levels of both *vg* and *sd* transcripts, but an almost normal *vg/sd* ratio, thus VG-SD activity is normal (fig 2). Consistently, no abnormal wing

phenotype could be detected. Therefore, the relative *vg/sd* stoichiometry, rather than absolute *vg* and *sd* expression levels, determines wing growth. Interestingly, it had been previously observed that *dap* homozygous mutant adult escapers display duplication of the wing margin (Lane *et al.* 1996), indicating a role of DAP at the D/V boundary. This phenotype could be linked to an enhanced proliferation due to the absence of CKI function. Moreover, D/V-specific over-expression of *dap* alters wing margin structures (Van de Bor *et al.* 1999). This *dap* over-expression triggers both ectopic expression of *sd* and subsequent impairment of VG-SD activity associated with a proliferation decrease (fig 3).The associated wing phenotype is clearly enhanced in *vg* heterozygous flies (fig 4), providing evidence that *dap* over-expression affects VG/SD stoichiometry and represses VG-SD activity in wing development. This reveals a model in which, in the wing pouch, cell proliferation down-regulation through cyclin/CDK inhibition by DAP, may be enhanced by an additive reduction of VG-SD proliferation function. Such a mechanism probably participates *in vivo* in the control of balanced wing growth.

dE2F1 overexpression in different vg backgrounds

Our results also demonstrate that dE2F1-DP regulates VG-SD: the *dE2F1* heterozygote displays a reduced *vg*/*sd* ratio due to a decrease in *vg* and an increase in *sd* transcripts, associated with reduced dimer activity, comparable to the vg^{null}/+ context (fig 2). Thus, *dE2F1* is required for *vg* normal expression. This supports the hypothesis that the slower proliferation observed in these contexts is linked to an imbalance in the dimer ratio.

Conversely, over-expressing *dE2F1-DP - P35*, in a vg^{83b27} hypomorphic mutant context, rescued expression of both *vg* and *sd* and normal VG-SD function, wing appendage specification and growth (fig 5). This was not observed in vg^{null} flies implying the necessity for *vg* sequences, but the second intron, missing in the vg^{83b27} mutant (Williams *et al.* 1994) . In addition, we ascertained that not all the genes triggering cell cycle progression or cell proliferation can induce *vg* expression. Neither ectopic expression of CYC E, which promotes dE2F1-induced G1/S cell cycle transition, nor the growth regulator Insulin receptor (InR) was sufficient to elicit VG expression and wing growth in the vg^{83b27} mutant (data not shown). These results demonstrate that *vg* induction is a prerequisite for vg^{83b27} wing pouch growth in response to dE2F1 activity.

In a vg^{+} genetic background, *dE2F1* over-expression induced only *sd*, disrupting VG/SD stoichiometry (fig 6). Consistently, at the D/V boundary, wing notching was observed (fig 4). Therefore, although *dE2F1* basically displays a positive role in proliferation, this *sd* induction in response to *dE2F1* over-expression was clearly associated with wing growth impairment. This effect was significantly weaker in a *vg* heterozygote background (fig 4), and a rescue of the wing phenotype was observed, supporting the hypothesis that VG/SD stoichiometry is restored. Therefore, *sd* induction by *dE2F1* depends on the *vg* genetic context. This indicates that the effects of over-expressing *dE2F1* differ depending on the growth-state of the wing pouch, which is tightly linked with the *vg* genotype.

Homeostatic regulations between dE2F1 and VG-SD

Clearly, feedback regulations rule growth of the wing disc. We have reported regulations in three different *vg* genetic contexts that can be analysed in the light of a homeostasis hypothesis. In the vg^{83b27} under-proliferative wing pouch, ectopic *dE2F1* expression coordinately increases *vg* and *sd* expressions in a positive feedback loop. This triggers VG-SD activity, and induces both cell proliferation and wing specification in the mutant. Conversely, no such crosstalk occurs in a correctly grown vg^{+} disc, where over-growth should be prevented. In this latter case, *sd* induction (VG/SD decrease) probably restrains the proliferation function of dE2F1. Consistently, wings were not overgrown, but notches were observed. This

phenotype was partially suppressed in a *vg* heterozygote background. As a whole, these results support the hypothesis that VG-SD/dE2F1 coordination tends to ensure normal wing growth and that the dimer does not trigger unrestricted cell proliferation in a vg^{+} context, since an excess in dE2F1 attenuates VG-SD function in a negative feedback loop. Thus, molecular interactions between *dE2F1*, *vg* and *sd*, display a clear plasticity depending on the *vg* genetic context.

Fine-tuning wing cell proliferation.

Establishing and maintaining homeostasis is critical during development. This is achieved in part through a balance between cell proliferation and death. In mammals *E2F1* and *p21*, the *dacapo* homolog, play a key role in this process. In the wing disc compensatory proliferation induced by cell death has been observed (Huh *et al.* 2004). However, the role of cell cycle genes in this process has not yet been established. How patterns of cell proliferation are generated during development is still unclear. It seems nevertheless likely that the gene responsible for regulating differentiation also regulates proliferation and growth (Skaer 1998). For instance, Hedgehog (HH) induces the expression of Cyclins D and E. This mediates the ability of HH to drive growth and proliferation (Duman-Scheel *et al.* 2002). In the same way, other data support a direct regulation of *dE2F1* by the Caudal homeodomain protein required for anterio-posterior axis formation and gut development (Hwang *et al.* 2002). Wingless (WG) also displays both patterning and a cell cycle regulator function during *Drosophila* development (Johnston & Sanders 2003).

Here we show that growth control in the wing pouch seems to be achieved through both positive and negative feedback regulations linking dE2F1 and VG-SD, but also *via* additive impairment of VG-SD by DAP. In fact, in a vg^{+} background, over-expression of both *dap* and *dE2F1* induces *sd*, impairs VG-SD and alters wing development. Nevertheless, clear opposite behaviours are observed in vg^{null}/+ flies where *dap*-induced nicks are enhanced, while those of *dE2F1* are partially rescued (fig 4). This highlights the functional discrepancy between these two types of feedback regulation. We suggest that *dap* expression inhibits cell proliferation through a process involving both Cyclin-CDK inhibition and VG-SD impairment in the wing pouch. On the other hand, we propose that *dE2F1* over-expression triggers an homeostatic response. It will either induce *vg* and *sd* to ensure proliferation (in a vg^{83b27} genotype), or decrease the VG/SD ratio in a vg^{+} context. In this latter genotype, down-regulation probably counteracts fundamental proliferative properties of dE2F1 and governs homeostatic wing disc growth.

At late third instar, wing discs display a Zone of Non-proliferating Cells (ZNC) along the wing pouch D/V boundary (O'Brochta & Bryant 1985). It has been shown that, although dE2F1-DP is expressed in this area, its proliferative function is inactivated late, because of RBF1-induced G1 arrest (Duman-Scheel *et al.* 2004); (Johnston & Edgar 1998). Accordingly, although expression of *vg* and *sd* presents a peak at the D/V boundary, in late third instar, VG-SD activity is decreased in D/V cells, and it was suggested to result from an excess of SD (Vaudin *et al.* 1999). Therefore, the ZNC setting may also reflect a VG-SD/dE2F1 coordinated dialogue that triggers a decrease in proliferation signals in this area.

Previous studies of homeostatic control of cell proliferation in the wing reported that, to some extent, over-expression of positive or negative cell cycle regulators only weakly affects the overall division rate (Reis & Edgar 2004). For instance, although *dap* over-expression alters dE2F1 function in G1-S cell cycle transition, it also promotes *dE2F1* expression and function in G2-M transition, preventing a decrease in the overall rate of cell division. Strikingly, cells seemed able to monitor each phase length and maintain cell cycle duration and normal proliferation in the wing pouch of the disc. Therefore, dE2F1 is a central component that enables cells to ensure normal proliferation in the wing disc and prevents imbalance in the process (Reis & Edgar 2004). The fact

that dE2F1 triggers quite different or opposite responses in vg^{+} or *vg* hypomorphic contexts suggests that the VG-SD/dE2F1 crosstalk plays a role in the same sort of homeostatic process that ensures entire wing growth.
We believe that such regulations are likely to reveal a precise physiological fine-tuning of *vg* and *sd* by cell cycle effectors, promoting an exquisite control of wing growth. Feedback loops between the developmental selector VG-SD and cell cycle effectors may stand for a control mechanism to guarantee that the tissue can sustain balanced growth and a reproducible size. Such a subtle mechanism, on a local scale, would correct the alterations in cell proliferation that may occur during development.

Experimental Procedures

Drosophila strains

The following strains were used : vg^{null} and *UAS-vg* (Paumard-Rigal *et al.* 1998); (Zider *et al.* 1998), *vg-GAL4* (gift from S. Carroll), vg^{83b27} (gift from J.Bell) vg^{BE}*-LacZ* and vg^{QE}*-LacZ* (Kim *et al.* 1996) (Williams *et al.* 1994), sd^{58} and the sd^{ETX4} *P[LacZ]* enhancer trap mutant that do not display any wing phenotype in the heterozygous state (Campbell *et al.* 1992), *UAS-sd* (Varadarajan & VijayRaghavan 1999), $dE2F1^{91}$ (Duronio *et al.* 1996), *UAS-E2F1, UAS-DP, UAS-P35* (Neufeld *et al.* 1998), dap^{04454} (de Nooij *et al.* 1996), *UAS-dap* (Lane *et al.* 1996), *hsp-GAL4*$_{db}$*-sd* (Vaudin *et al.* 1999). Heat shocks (38°C, 30 min) were performed 36 hours before puparium formation and wing discs were stained 24h after the heat shock. All other stocks come from the Bloomington *Drosophila* Stock center (Indiana University)

Scanning Electron Microscopy (SEM)

Adult flies were anesthetized with ethyl acetate (Sigma), mounted on stages and covered with a 40 nm layer of gold salts. Preparations were observed under a JEOL JSM 6100 Scanning Electron Microscope. Images were acquired using Genesis software.

Histology

Images were acquired with a DMR (Leica) microscope and processed with Adobe Photoshop. 5-Bromo-2'-deoxyuridine (BrdU) and 4'-6-Diamidino-2-phenylindole (DAPI) labeling of imaginal discs were performed as described (Delanoue *et al.* 2004). Antibody staining: rabbit anti-phospho-Histone H3 antibody (Upstate Biotechnology), anti-VG (gift from S. Carroll), anti-DAP (gift from C. Lehner) anti-βgalactosidase (Biodesign) antibodies, and mouse anti-βgalactosidase (Jackson Immuno Labs), anti-CT (Developmental Studies Hybridoma Bank (DSHB), University of Iowa) antibodies. We assessed the activity of the VG-SD transcriptional reporter strain 5 times independently, using both anti-βgalactosidase antibodies or the X-gal enzymatic reaction.

Quantitative Real time RT-PCR

Total RNA of 20 wing discs from third instar larvae was isolated by using tri reagent (Molecular Research Center Inc.) according to the manufacturer's instructions. One μg of RNA was used for reverse transcription into cDNA according to the supplier's instructions (Roche). Real time PCR was conducted in a Light Cycler (Roche) with 45 cycles (15 sec 95°C, 15 sec 60°C for *vg, RP49* - 65°C for *sd*, 15 sec 72°C) 1 μl cDNA, and PCR reaction mix from Roche. The following primers were used for amplification: *sd*: aatattgcaagtaatgagggccc / gacggtataatgtgatgggtggtg ; *RP49* : ccgcttcaagggacagtatctg / cacgttgtgcaccaggaactt ; *vg* : cggcccactatggttcctatg / agcctgaggagactgccgtact. Differences in cDNA concentrations were adjusted by normalizing to RP49. For each gene, values were averaged over at least three independent measurements. The expression levels were calculated relative to the wild type genotype (w^{1118}) (the expression in this genotype was set to 1). Two independent RNA isolation experiments were performed for all genotypes and means were calculated.

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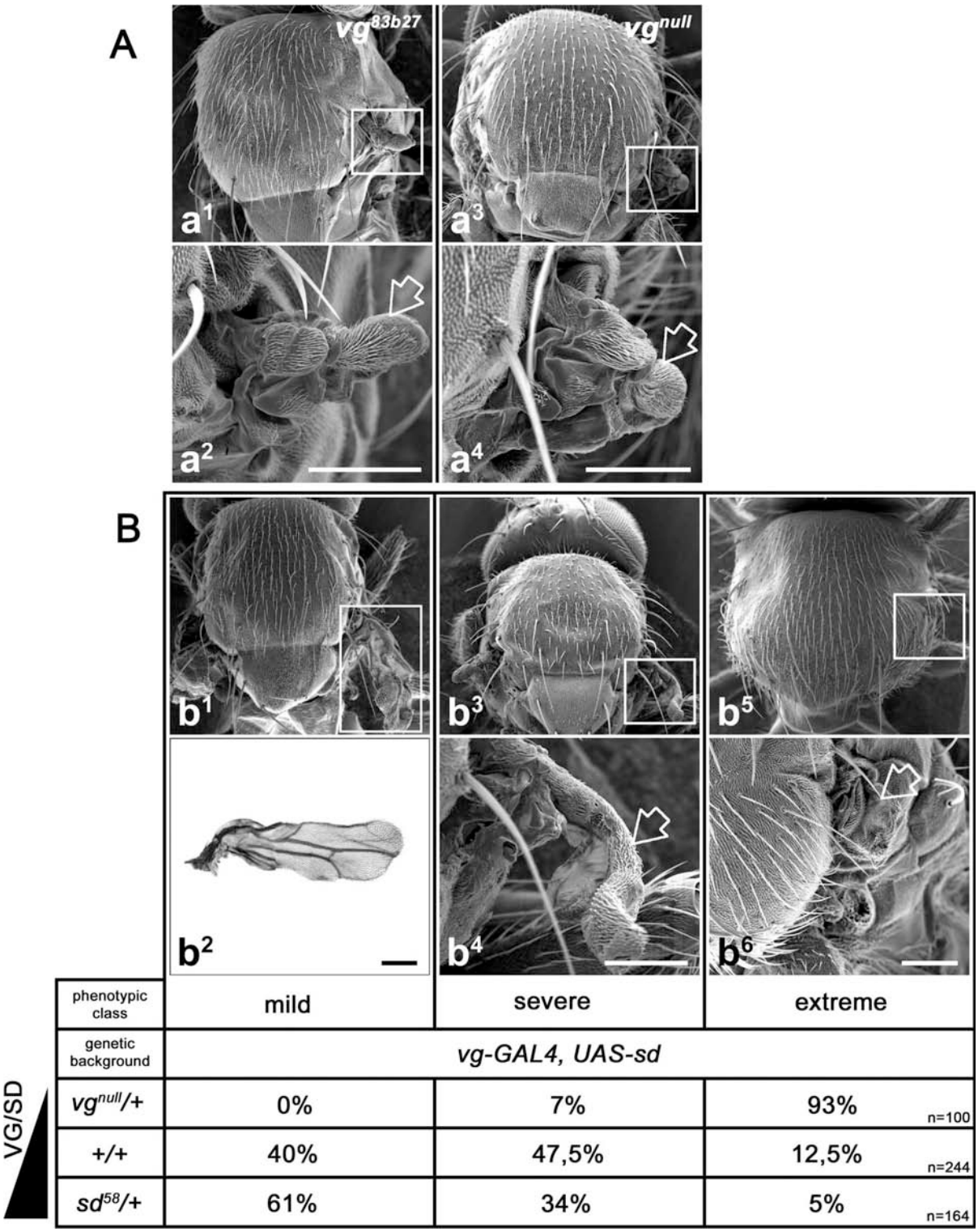

	phenotypic class	mild	severe	extreme	
	genetic background	*vg-GAL4, UAS-sd*			
VG/SD	vg^{null}/+	0%	7%	93%	n=100
	+/+	40%	47,5%	12,5%	n=244
	sd^{58}/+	61%	34%	5%	n=164

Figure 1 : VG-SD stoichiometric ratio is critical for wing growth.

(A) Thoraxes of vg^{83b27} (a1) and vg^{null} (a3) mutants display no growth of wing blade tissue, although some hinge structures are still present (arrows in a2 and a4). (B) *vg-GAL4* driven over-expression of *sd* in sd^{58}/+ ; +/+ or vg^{null}/+ genetic backgrounds providing a sequential decrease in VG/SD ratio. Wing phenotypes are classified into three distinct categories. Mild (b1) wings are significantly reduced, few veins and cross-veins are still distinguishable, (b2) Photonic magnification of a similar wing. Severe: (b3 and b4) Wing blade growth is severely impaired (arrow). Extreme: (b5 and b6) Thorax flight appendages are entirely missing. No hinge structure is recovered (arrow). All pictures except (b2) are Scanning Electron Micrographs (SEMs) and scale bars refer to 100mm. Phenotypic class percentages are presented for each genetic background. When the VG/SD ratio is decreased, a shift toward extreme phenotypes is observed.

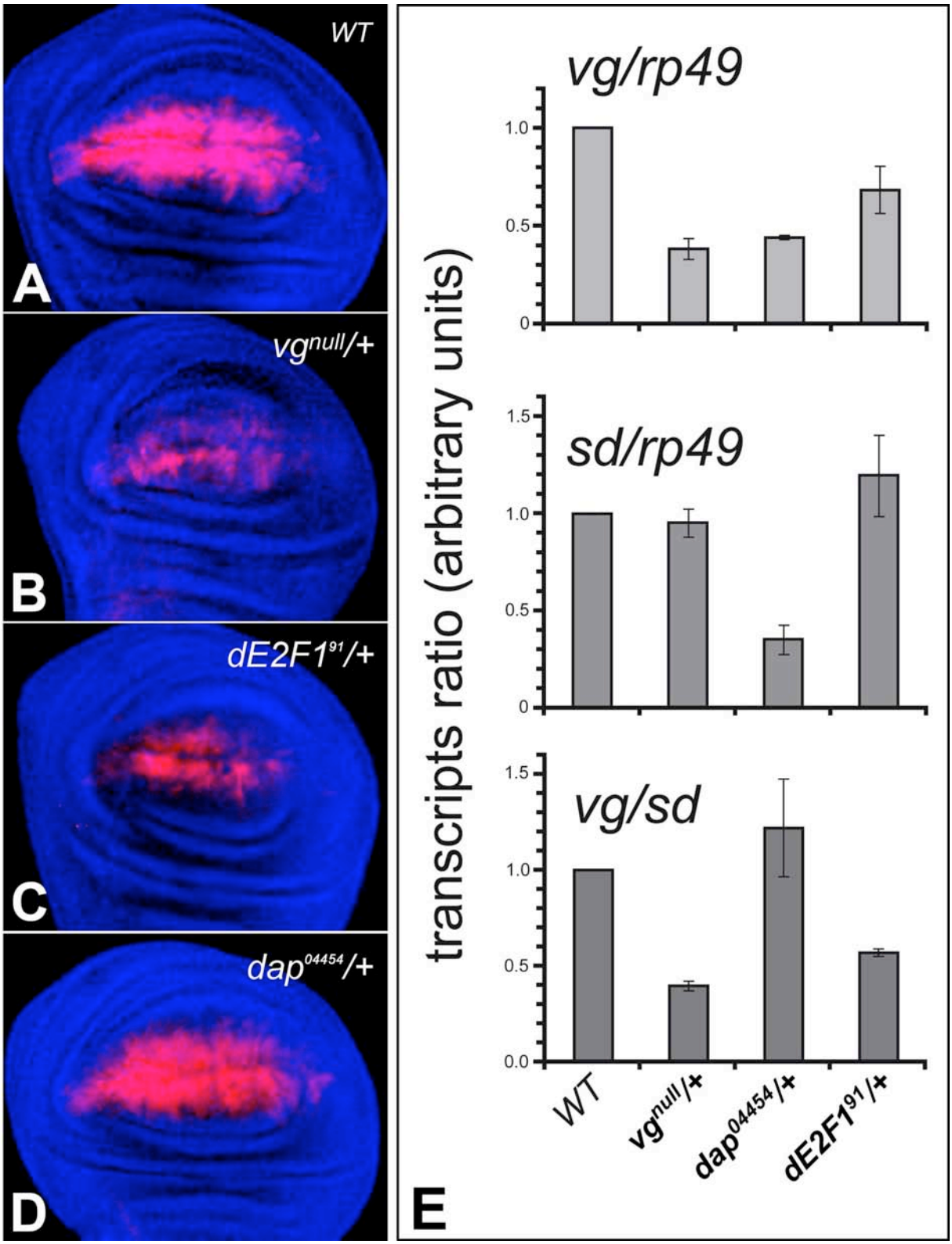

Figure 2 : VG-SD transcriptional activity depends on cell cycle gene expression.
All third instar wing discs are orientated with the posterior compartment to the right and the ventral one to the top. These *hsp70-GAL4db-sd, UAS-lacZ* discs heat shocked and stained with anti-bgalactosidase antibodies (red) and the nuclear dye DAPI (blue), 24 hours later. Compared to the control (A), a significant decrease in VG-SD activity can be observed both in vg^{null}/+ (B) and $dE2F1^{91}$/+ (C), but not significantly in dap^{04454}/+ discs (D). (E) Quantitative real time RT-PCR was performed in wing discs of these 4 genotypes. *vg* and *sd* transcripts were measured, adjusted by normalizing to *RP49*, and set to 1 in WT control discs. *vg/sd* transcripts ratio are shown. In vg^{null}/+ and $dE2F1^{91}$/+ wing discs, the *vg/sd* ratio is decreased to about a half of the value observed in control and other genotypes.

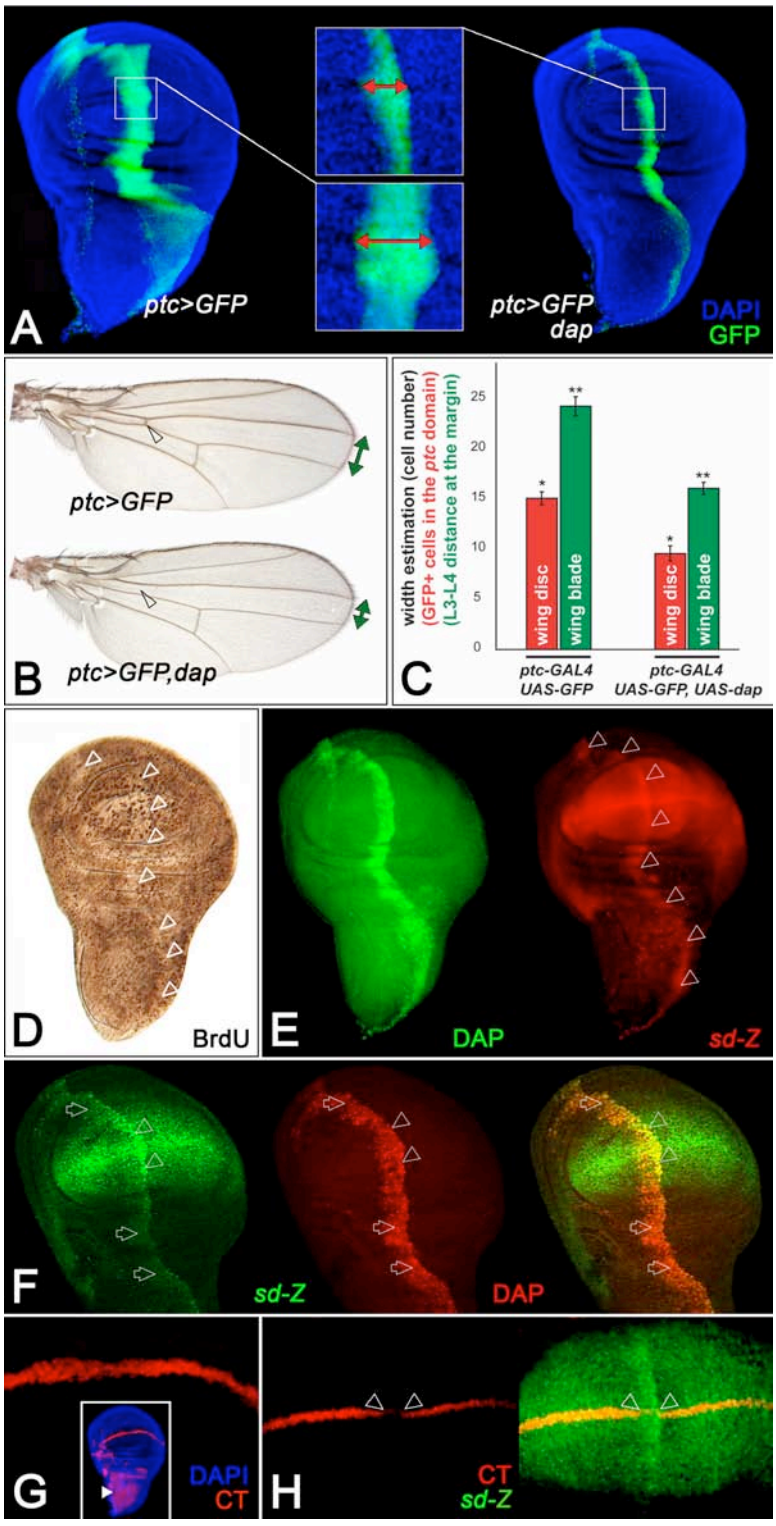

Figure 3: Over-expression of *dap* induces ectopic expression of *sd*.
(A) *ptc-GAL4, UAS-GFP* third instar wing discs over-expressing *dap* or not. The *ptc* domain width (red arrows) is estimated by the number of nuclei (DAPI) in the GFP+ domain at the margin. (B) Adult males wing blades of the same genotypes. Margin width (cells) between L3 and L4 veins (green arrows) is estimated by the number of bristles. The anterior cross-vein of *dap* over-expressing wings is often missing (arrowhead). (C) The widths of the wing disc *ptc* and adult L3-L4 at the margin measured in flies expressing *UAS-dap* or not, are presented on the graph. n=30 for each experiment. Student's test : $p<0.001$ for both * and ** tests. (D-F, H) sd^{ETX4}/+ ; *ptc-GAL4, UAS-dap* wing discs. (D) BrdU incorporation reveals a decrease in S phase cells along the *ptc* domain (arrowheads). (E) Endogenous DAP is expressed homogeneously in the wing disc, while over-expressed DAP is detected along the *ptc* domain. *sd-lacZ* ectopic expression is induced, along the *ptc* domain, in response to *dap* (arrows). (F) Confocal sections. *sd* is strongly over-expressed at the posterior sharp edge of the *ptc* domain in the pouch (arrowheads), while ectopic expression outside the wing pouch is weaker (arrows). (G-H) are wing pouch magnification. (G) wild type D/V specific CT expression (open arrowhead) is magnified. (H) CT expression is lost from cells over-expressing *sd* (arrowheads) in response to DAP.

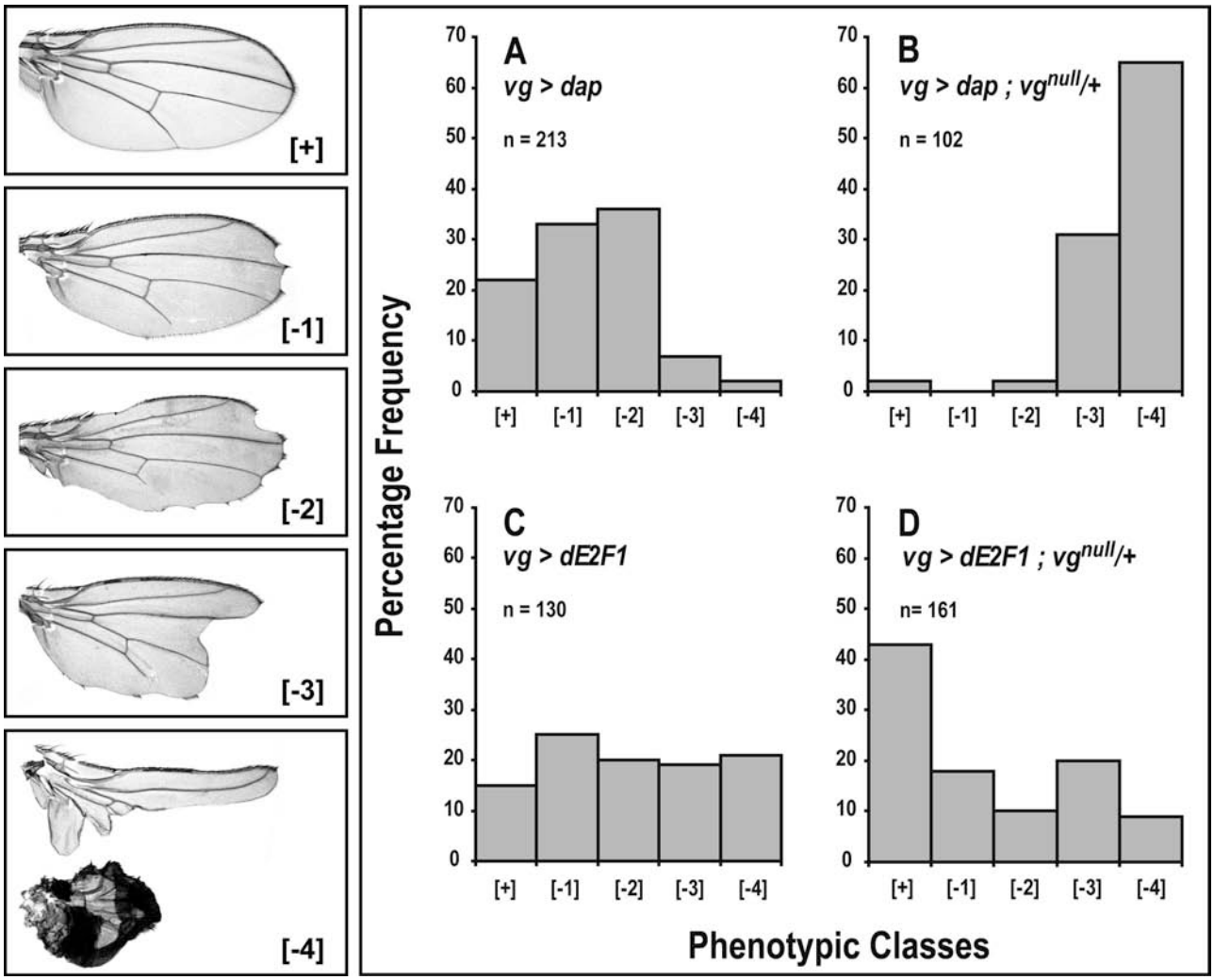

Figure 4: Effects of cell cycle gene expression in different *vg* backgrounds.
dap (A) or *dE2F1+DP+P35* (C) are over-expressed along the D/V boundary of the disc (*vg-GAL4* driver). Expression of P35 prevents cell death associated with dE2F1-DP over-expression. The defects in wings range from mild nicks at the tip of the wings to strong notching and wing size reduction and were quantified using an arbitrary scale of [-1] to [-4]. [+] indicates normal wings. In a vg^{null}/+ context, wing phenotypes associated with *dap* over-expression are enhanced (B), while *dE2F1*-induced nicks are partially rescued (D). Data in each plot are based on an analysis of at least 102 wings.

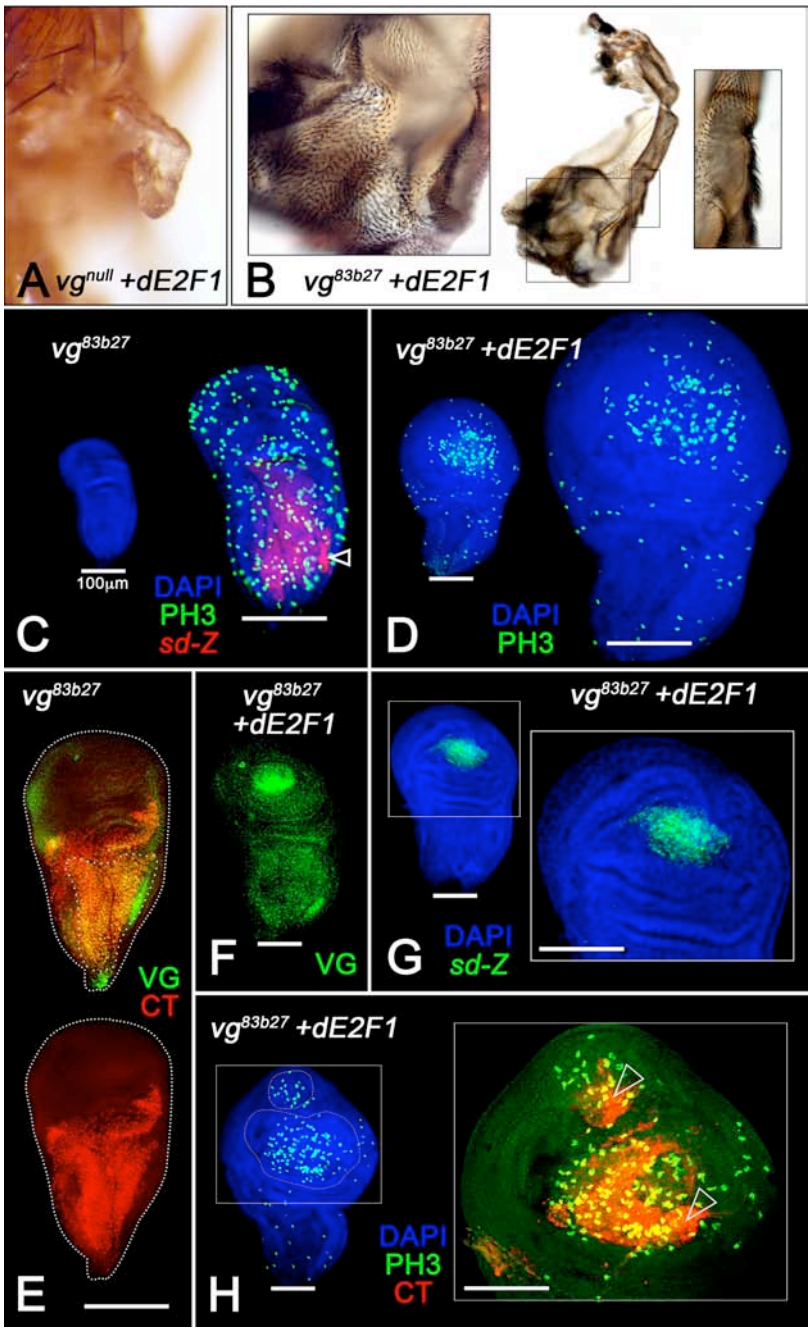

Figure 5: *vg-GAL4* driven expression of *dE2F1* rescues wing development in a vg^{83b27} context. *dE2F1* over-expression according to the *vg-GAL4* driver is achieved with the use of a *UAS-dE2F1, UAS-DP, UAS-P35* strain. (A) *dE2F1* over-expression, in a vg^{null} mutant, triggers wing hinge growth, while no wing blade structure is observed (compare with fig 1A). (B) In a vg^{83b27} mutant, *dE2F1* rescues wing appendage growth. Note the presence of wing blade features such as bristles, wing veins and margin (insets). (C-H) Third instar wing discs stained with the nuclear dye DAPI and the mitotic marker anti- phosphohistone H3 (PH3) antibodies while *sd* expression is monitored in sd^{ETX4}/+ females (C) In vg^{83b27} mutants, *sd* is weakly expressed in myoblasts (arrowhead in the *notum*) but not in the putative wing pouch, where proliferation is weaker (open arrowhead). (D) *dE2F1* expression rescues vg^{83b27} wing disc growth. Proliferation is specifically triggered in the wing pouch that is recovered. (E) In vg^{83b27} discs, CT is expressed in all myoblasts, whereas VG is only weakly expressed in a subset of these adepithelial cells (dotted line). *dE2F1* expression in a vg^{83b27} wing disc rescues expression of both VG (F) and *sd* (G) in the wing pouch. (H) *dE2F1* also triggers CT expression (arrowheads) as visualized in the two proliferating wing pouches (surrounded). All scale bars refer to 100µm.

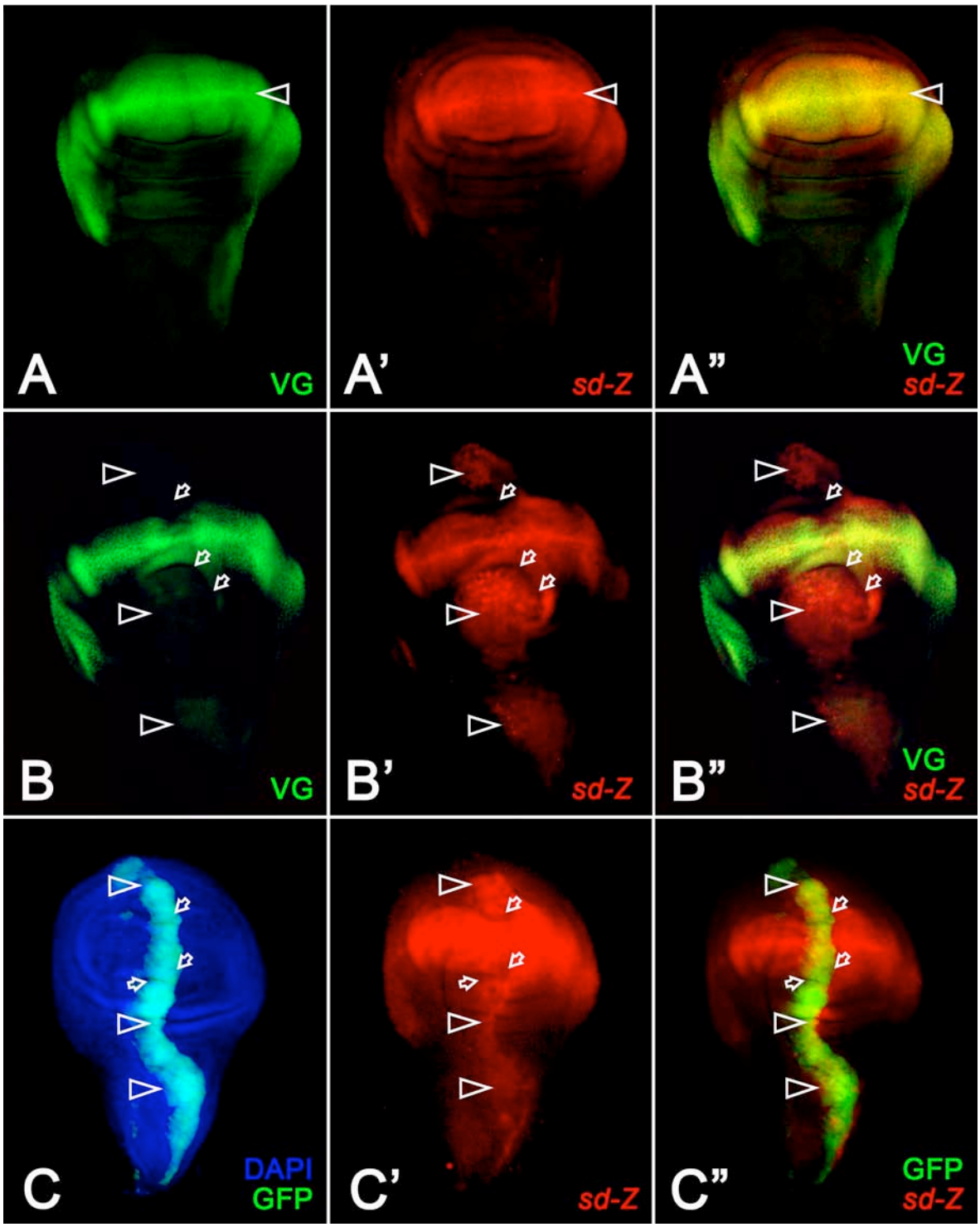

Figure 6 : Regulation of *sd* expression by *dE2F1*, in a vg^{+} background.

Third instar wing discs. *sd* expression was monitored in sd^{ETX4}/+ females. (A, A', A") Expression of VG and *sd-lacZ* colocalize mainly in the wing pouch, High levels are observed along the D/V boundary (arrowheads). (B-C) *ptc-GAL4 ; UAS-GFP UAS-dE2F1, UAS-DP, UAS-P35* discs. (B, B', B") *sd* ectopic expression is induced along the *ptc* domain, in response to *dE2F1* expression (arrowheads), whereas VG is not. Induction of *sd* in the notum is uncertain, since both *vg* and *sd* are weakly expressed in myoblasts in a wild type disc. *dE2F1*-induced proliferation promotes epithelium folding (arrows). (C, C', C") DAPI staining highlights the folds around the pouch (arrows) Note the ectopic expression of *sd* (arrowheads) along the entire *ptc* domain marked by GFP expression.